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14. ABSTRACT

We will generate a novel approach for detection and therapy of advanced disseminated breast cancer based on three fundamentally novel discoveries and concepts. The first critical component exploits the discovery of a novel antibody, which targets phosphatidylserine (PS), expressed on tumor vasculature and stimulates recruitment of host defense cells to attack the vasculature. In collaboration with Peregrine Pharmaceuticals, this agent has been chimerized and is being developed for clinical trials as bavituximab (formerly named Tarvacin™). Normally, PS exclusively resides on the cytosolic leaflet of the plasma membrane. However, in tumors PS becomes externalized and provides a viable target. The agent not only targets various tumors, but also induces vascular damage and tumor regression with minimal accompanying toxicity. The second key component is the identification of diverse arsenic radionuclides suitable for imaging based on positron emission tomography (PET; ^{72}As, ^{74}As) and radio immunotherapy; ^{77}As). Of equal significance was the discovery that antibodies derivatized with N-succinimidyl-S-acetylthioacetate (SATA) could be effectively labeled forming viable products of high specific radiochemical and biological activity. Thirdly, bringing together the pharmacological and radiochemical expertise and identifying animal tumor models and imaging instrumentation to facilitate collaboration, progress, and synergy. This project will permit a single agent (bavituximab) to serve as the foundation for tumor detection, dosimetry, and therapy based on differential properties of arsenic radionuclides, but exploiting a single chemistry.

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Introduction

We proposed to develop and evaluate a novel therapeutic approach to breast cancer, which may be effective at any stage of tumor development, but which would be most relevant to advanced disseminated disease. We will combine a novel tumor vascular target with new radioarsenic chemistry to generate a platform technology encompassing tumor detection, dosimetry, and radio immunotherapy (RIT). Recently, Thorpe *et al.* identified phosphatidylserine (PS) as a new vascular target and generated an antibody (3G4) (1-3). It is thought that PS is exposed on tumor vasculature due to stress conditions in the tumor microenvironment. Externalized PS is a desirable marker for tumor targeting for several reasons: 1) PS is specifically and abundantly expressed on tumor vascular endothelium; 2) it is present on a high percentage of tumor endothelial cells in various solid tumors; 3) it is absent from normal vessels; 4) it is on the luminal side of tumor endothelium, which is readily accessible for targeting drugs. Since this target should be ubiquitous among tumors, we believe PS can form the foundation for a potent new anti breast cancer therapy. 3G4 has been developed as Tarvacin (a human chimerized monoclonal antibody, which has recently been given the USAN name bavituximab) by Peregrine Pharmaceuticals, who now have an IND to initiate Phase 1 clinical trials based on direct therapeutic efficacy (4). However, at much lower doses, we believe Tarvacin may be used to detect breast tumors using positron emission tomography (PET) and treat them by radio immunotherapy based on arsenic radionuclide ligands. Such systemic therapy should effectively target disseminated disease, yet provide highly localized tumor specific toxicity.

To efficiently apply bavituximab to breast cancer, we will use radioisotopes of arsenic. Multiple isotopes are available with diverse characteristics making them suitable for nuclear medicine imaging and radio immunotherapy. Arsenic-74 has a long half-life (~18 days) and positron emitting activity making it suitable for PET in animal models, while ^{72}As would be suitable in the clinic (5-7). Meanwhile, ^{77}As is a pure β^-emitter, potentially suitable for radio immunotherapy (RIT). Jennewein and Rösch (consultants) have developed novel chemistry to effectively isolate arsenic radionuclides from irradiated germanium targets and generate ligands of radio arsenic on antibodies to target breast tumors. To date dosimetry has been a major obstacle to effective

RIT, since the pharmacokinetics and accumulation in tumors of mAbs can be highly variable. We believe we can establish a novel approach for tumor detection (both primary and metastases), which will allow analogous chemistry for targeted radio immunotherapy.

It should be noted that the antibody Tarvacin has now been formally named bavituximab.

Body and Progress

Phase 1 Optimize Tarvacin labeling with arsenic radionuclides for imaging, biodistribution, and radio immunotherapy.

Task 1 Months 1-4- continued in year 3 as we seek to optimize procedures.

a) Tarvacin (a human chimeric anti phosphatidylserine (PS) monoclonal antibody (available by collaboration with Peregrine Pharmaceuticals) will be derivatized with N-succinimidyl S-acetylthioacetate (SATA), labeled with the radionuclide ^{77}As, and evaluated for activity.

Arsenic-77 is a 100% β^- emitter which could be a possible new candidate in radionuclide therapy, *e.g.*, intravascular radiation therapy (IVRT), or radio-immunotherapy (RIT). This radionuclide can be produced from natural germanium in nuclear reactors through the neutron induced nuclear reaction, ^{76}Ge (n, γ) ^{77Ge}, where ^{77}Ge decays to ^{77}As with a half life of 11.3 h. Dr. Sun negotiated supplies of ^{77}As from the nuclear reactor facility at the University of Missouri (MURR) courtesy of Dr. Cathy Cutler. During years 2 and 3 our radiochemist, Dr. Guiyang Hao has continued development of radiochemistry. Our consultant Dr. Jennewein visited Dallas to train Dr. Hao in labeling procedures. The efforts have led to a manuscript submitted to *Applied Radiation Isotopes* (appended) and further manuscripts are in preparation. Recent methods are described as follows, developing further on the experience reported for Year 2. Certain critical parameters have been identified, such as critical pH adjustments during labeling procedures to avoid premature oxidation of AsI_3.

Recently, Jennewein *et al*. (2005) described a method to separate no-carrier-added (nca) arsenic iodide, [^{77}As]AsI_3, from an irradiated GeO_2 matrix in a hydrofluoric acid medium using a polystyrene based solid–phase extraction system (ENV cartridge) and KI solution. [^{77}As]AsI_3 was prepared by following the same

procedure. Briefly, irradiated GeO_2 targets were dissolved in 5 ml conc. HF at room temperature for 1 h. Subsequently, 20 mg NaI was added into 1 mL HF solution containing $^{77}Ge/^{77}As$ and stirred for 10~30 min. The mixture was transferred to an ENV solid phase extraction cartridge. The ENV cartridge was preconditioned with 5 ml of MeOH, 5 ml H_2O and 5 ml conc. HF containing 50 mg NaI. The nca $^{77}AsI_3$ was fixed to the solid phase, while the macroscopic $[GeF_6]^{2-}$ was eluted quantitatively with the mobile phase. After the fixation of $^{77}AsI_3$, excess HF was removed with a dry N_2 flow over the cartridge for 1 hr. The elution of $^{77}AsI_3$ was performed with anhydrous ethanol. In year 1 and 2 we achieved maximum yields of 10 to 15%, which has been improved during the past year up to 23% (without decay correction).

All reagents and solvents were purchased from Pierce, Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise noted. As-77 was purchased from University of Missouri, Columbia (Columbia, MO). Cu-64 was purchased from University of Wisconsin (Madison, WI). Milli-Q water (18 M Ω-cm) was obtained from a Millipore Gradient Milli-Q water system (Billerica, MA). Centricon filters (YM-10: MWCO 10 KDa; YM-30: MWCO 30 KDa) were purchased from Millipore, dialysis tubing from Spectrum Laboratories (Rancho Dominguez, CA). The desalting columns (D-Salt Excellulose Desalting Columns, 5×5 mL) were purchased from Pierce. Radio-HPLC analysis was performed on an Agilent 1100 Series system equipped with LDC/Milton Roy UV monitor III at 254 nm and a 'Gabi' NaI radiation monitor from Raytest.

Preparation of $^{77}AsI_3$

Method 1. Irradiated germanium oxide targets were dissolved in 5 ml conc. HF at room temperature for 1 h. Subsequently, sodium iodide was added into 1 mL HF solution containing $^{77}Ge/^{77}As$ and stirred for 10 min. The amount of NaI was used with a small spatulas amount. The mixture was transferred to an ENV solid phase extraction cartridge. The ENV cartridge was preconditioned with 5 ml of MeOH, 5 ml H_2O and 5 ml conc. HF containing NaI. The nca $^{77}AsI_3$ was fixed to the solid phase, while the macroscopic $[GeF_6]^{2-}$ was eluted quantitatively with the mobile phase. After the fixation of $^{77}AsI_3$, excess HF was removed with a nitrogen-flow over the cartridge for 1h. The elution of $^{77}AsI_3$ was performed with 500 µL of ethanol.

The whole process to p repare $^{77}AsI_3$ according the m ethod of Marc Jen newein is shown in Fig. 1. $^{77}AsI_3$ retained in ENV cartridge very well even after around 5 h N_2 flow dry. During the fina l elution of cartridge, water should be avo ided because the hydrolysis of $^{77}AsI_3$ easily occurred. Therefore, we used ethanol to elu te the cartridge. Because the first 2 drops of ethanol eluent contained a lot of I2, it was collected separately and the left 0.5 mL ethanol eluent would be used for the following radiolabeling reaction.

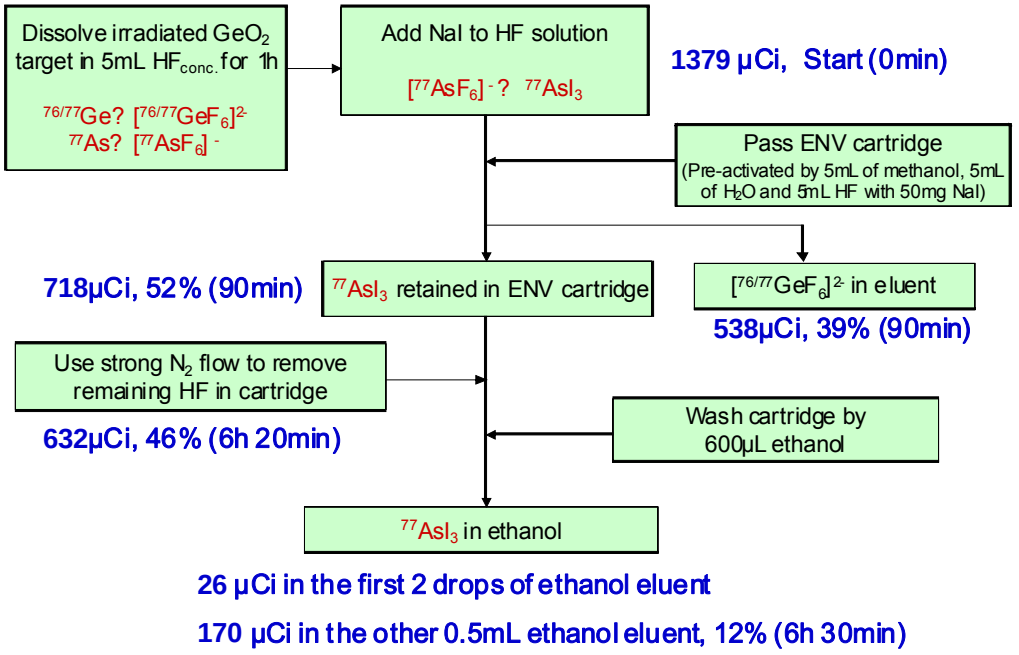


Figure 1. Process of preparing $^{77}AsI_3$ (typical results in year 2)

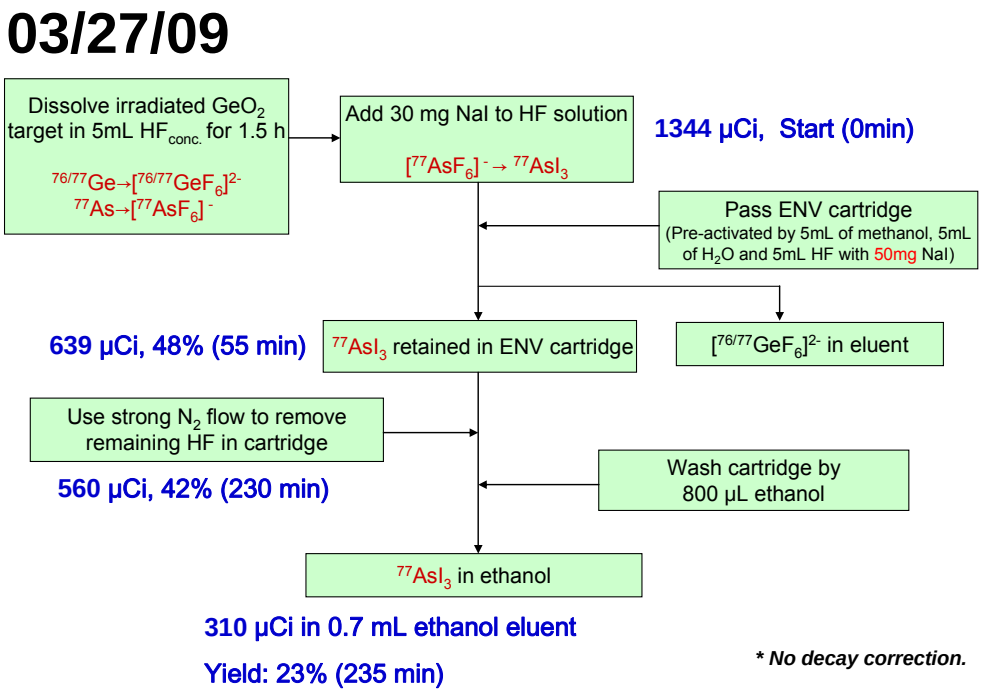


Figure 2. Process of preparing $^{77}AsI_3$ (enhanced yield achieved in results in year 3)

Conjugation and Radiolabeling of Bavituximab by $^{77}AsI_3$

Proteins in cluding bavituxim ab and rituxan were SATA modified according to th e protocol of Pierce Endogen. Deprotection of the sulfh ydryl groups of the antibody derivative using hydroxylam ine was done directly before the labeling. The num ber of free th iol groups per antibody m olecule was m easured using Ellman's reagent and b y com parison with cys teine-based s tandards. Th iolated pro tein in PBS was com bined with the nca $^{77}AsI_3$ solution at 37ºC for 30 m in. $^{77}AsI_3$ couples to one -SH functionality with elim ination of HI. Quality con trol of the an tibody labeling was done by HP LC and TLC (thin layer chro matography). The HPLC column was a Bio Suite™ 125 10µm SEC, 300 ×7.8 mm a nd 0.1M PBS + 0.1 M NaCl was used as solvent at a flow of 1 mL/min. ITLC was done on I TLC paper with 10 m M PBS as eluent. The labeling yields varied fro m 0~47%.

During the addition of $^{77}AsI_3$ ethanol solution to the thiolated protein solution, several agents were tried to remove excess I_2 in $^{77}AsI_3$ ethanol solution. Although TCEP could gi ve m uch higher labeling efficiency (~70%), it could break the S-S bonds in the protein and impair the bio-activity. $Na_2S_2O_5$ and Na_2SO_3 could remove excess I_2 efficiently like TCEP. The following labeling yi elds really depend on the am ount of thiolated proteins ad ded. Hydroxylam ine, tyrosine, 4 -hydroxyphenyl acetic acid, and $Na_2S_2O_3$ didn't work for this purpose. For *in vivo* study, high sp ecific activity of labeled protein is ess ential. However, less protein will le ad to lower labeling yields (<5%) insufficient for injection. Therefore, highly efficient labeling conditions of $^{77}AsI_3$ to th iolated prote in would be the m ost critical fac tor for future use.

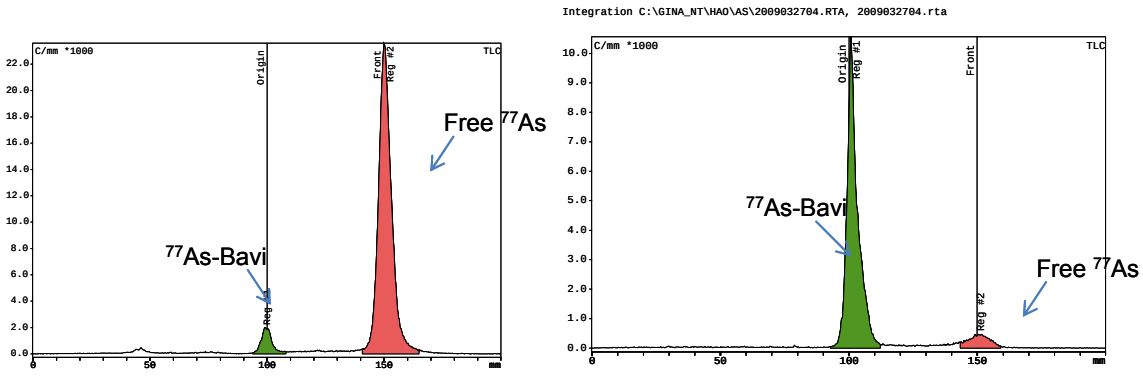


Figure 3 Removal of free ^{77}As. Trace at left indicate 7% labeled material before purification which was enhanced to 92% after purification on PD-10 column and concentration with Amicon centrifuge tube yielding 10 µCi ^{77}As-Bavituximab.

Various molar ratios of SATA to bavituximab were tested ranging from 5 to 1 to 15 to 1. The best labeling yield was found for 15 t o 1, although 10 to 1 gave poorer result that 5 to 1. Using less **Na_2SO_3** gave better yields as shown in Table 1

Table 1

Ratio	Bavi-SH Conc. (mg/mL)	$Na_2S_2O_5$ (mg)	Bavi-SH (mL)	$^{77}AsI_3$ (mL)	Yield(%)
5:1	1.17	0.54	1.2	0.1	15
10:1	1.18	0.54	1.2	0.1	7
15:1	1.11	0.54	1.2	0.1	17
Ratio	**Bavi-SH Conc. (mg/mL)**	**Na_2SO_3 (mg)**	**Bavi-SH (mL)**	**$^{77}AsI_3$ (mL)**	***Yield(%)***
5:1	1.17	0.36	1.2	0.1	16
10:1	1.18	0.36	1.2	0.1	12
15:1	1.11	0.36	1.2	0.1	25

Biodistribution of ^{77}As-Bavituximab

All anim al studies were perform ed in com pliance w ith gu idelines se t by the UT Southwester n Anim al Studies Committee. The injec tion doses were prepared by diluting the purified ^{77}As-Bavituximab with 10 mM PBS buffer and add 1 .2 m L of 2.1 m g/mL β2-glycoprotein I. Breast tum or-bearing m ice (2 weeks post implantation) were injected with 100 µL of ^{77}As-Bavituximab (~3 µCi/mouse) and 0.2 m g β2-glycoprotein via the tail vein. The anim als were sacrificed at 24 and 69 h post-injection (p.i.). Organs of interest (blood, lung, liver, kidney, spleen, muscle, fat, hear t, stom ach, sm all in testine, large intestine, b one, bra in, tail, and tumor) were removed, weighed, and counted. Standards were prep ared and counted along with the sam ples to calculate the percent injected dose per gram tissue (%ID/g) and percent injected dose per organ (%ID/organ). Just before dissection, the autoradiography was done.

Autoradiography of ^{77}As-Bavituximab

Autoradiography was performed on a PerkinElmer Cyclone storage phosphor system with OptiQuant software (Waltham, MA). The animal model was established by subcutaneous injection of a cell suspension into the right back flank of mice. During the study, each mouse received ~5 μCi of the ^{77}As-Bavituximab intravenously (n=2). The mice were anesthetized and laid dorsally on the phosphate plate for 1 hr of exposure before sacrifice

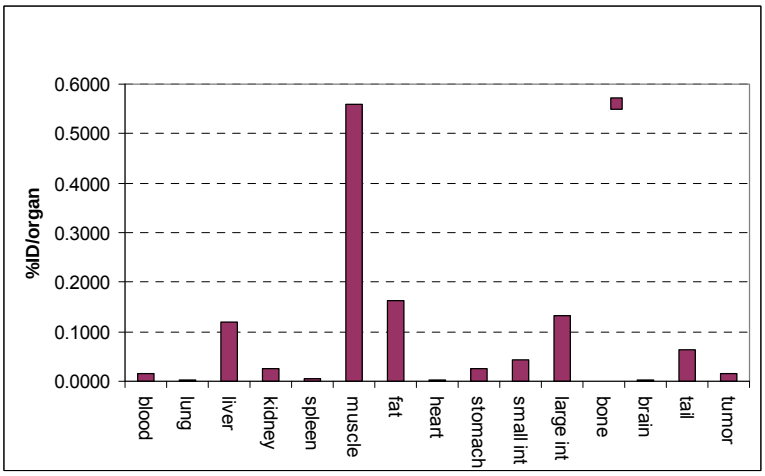


Figure 4. %ID/organ values of ^{77}As-Bavituximab in breast tumor-bearing mice at 24 hr p.i. (n=2)

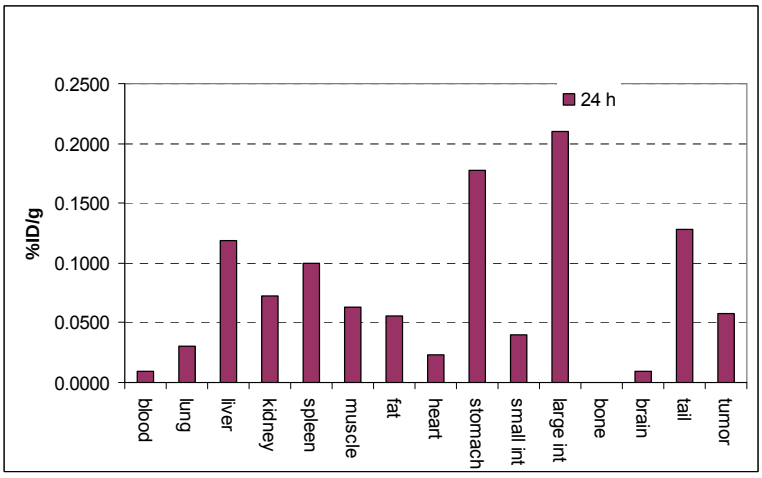


Figure 5. %ID/g values of ^{77}As-Bavituximab in breast tumor-bearing mice at 24 hr p.i. (n=2)

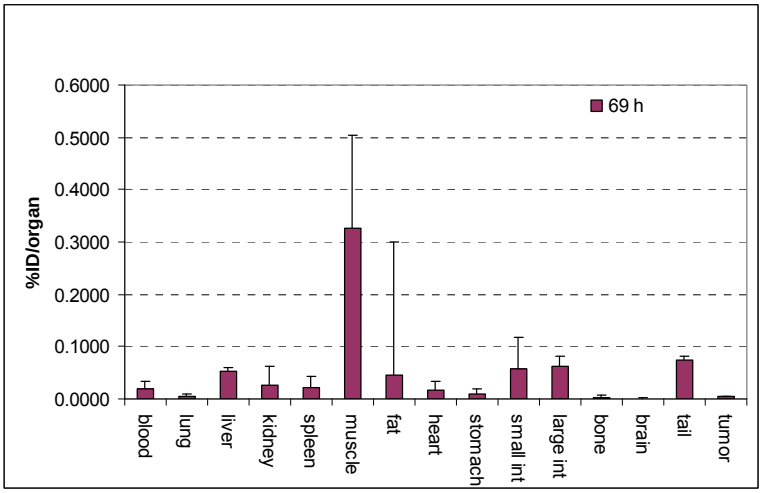


Figure 6. %ID/organ values of ^{77}As-Bavituximab in breast tumor-bearing mice at 69 hr p.i. (n=3)

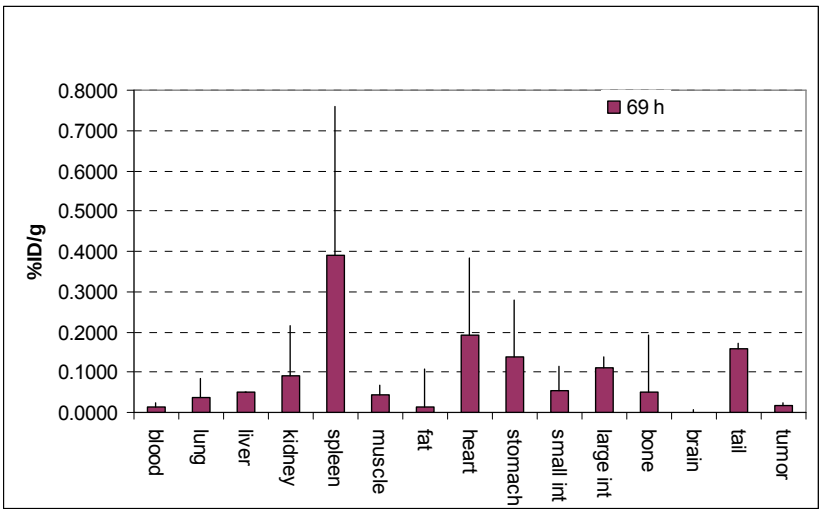
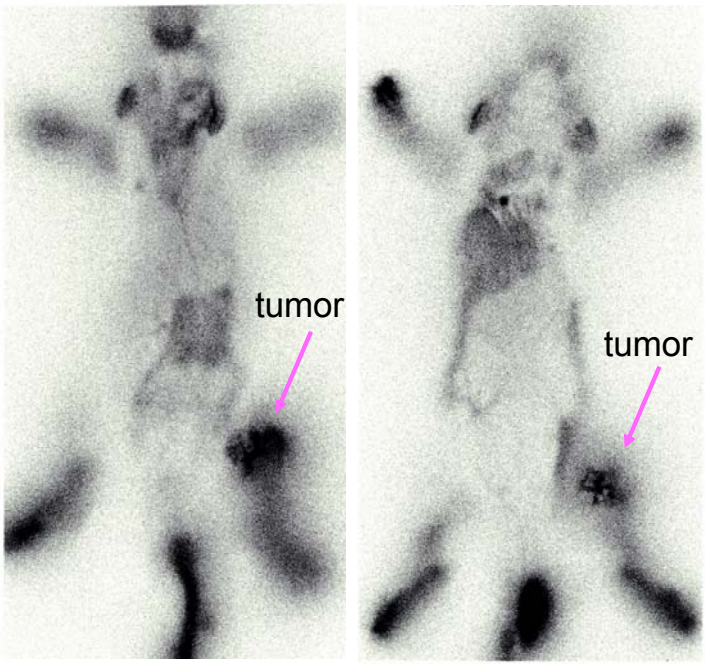


Figure 7. %ID/g values of ^{77}As-Bavituximab in breast tumor-bearing mice at 69 hr p.i. (n=3)



70h p.i. As-Bavituximab 24h p.i. As-Rituxan

Figure 8. Autoradiography of ^{77}As-Bavituximab in breast tumor-bearing mice at 24 h and 69 h p.i.

Given initial difficulties with effective labeling of antibody with ^{77}As we have also tested derivatization with 64-Cu. This could provide a parallel approach since copper has isotopes suitable for both PET and radioimmunotherapy (^{64}Cu and ^{67}Cu) just as proposed in our project for ^{74}As (or ^{72}As) and ^{77}As. Initial data look very promising as the foundation for an alternate approach.

Preparation of DOTA-Bavituximab

The preparation of the DOTA-Bavi tuximab conjugate is straightforward according to the well estab lished method. In order to remove the sm all molecules and dimm ers, the conjugate was purified by HP LC with size exclusion column. The fractions were collected and concentrated for radiolabeling.

Radiolabeling of Bavituximab by ^{64}Cu

^{64}Cu-DOTA-Bavituximab was labe led in 50 -60% lab eling yields and 98% radioc hemical purity after Bio - Spin column purification. The labeling yields can be in creased to m ore than 95%, if more DOTA-Bavituxim ab is added into ^{64}Cu solution.

Biodistribution of ^{64}Cu-DOTA-Bavituximab: It was shown in Fig. 9 that ^{64}Cu-DOTA-Bavituximab had hi gh tumor uptake (6.6 %ID/g) at 24 hr p.i. The uptake in liver, kidney, and other normal tissues except spleen w as low. More time point experiments are expected.

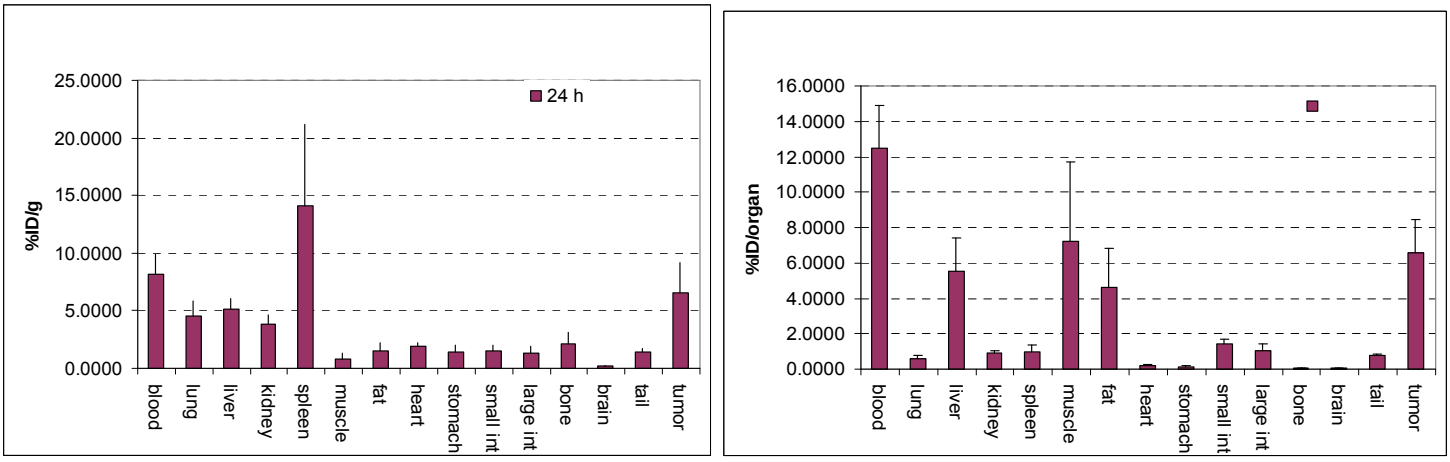


Figure 9. %ID/organ values of ^{64}Cu-DOTA Bavituximab in mice bearing breast tumor at 24h p.i. (n=3)

Autoradiography of ^{64}Cu-DOTA-Bavituximab

We can see the tum or clearly after 1h p.i. The tum or wa s in large s ize because these m ice had already been cultured for around 5 weeks. Mi cro-PET/CT research with ^{64}Cu-DOTA-Bavituximab in breast cancer model can be planned.

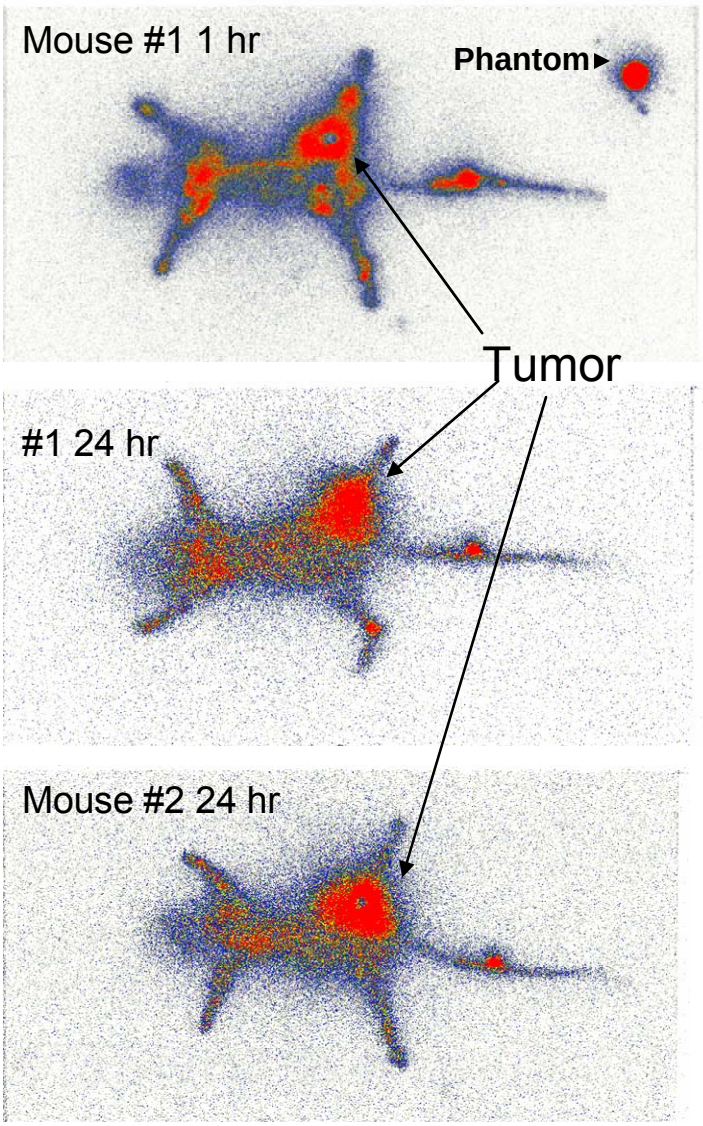


Figure 10. Autoradiography of ^{64}Cu-DOTA-Bavituximab in tumor mice:

Top mouse #1 1 hr p.i.

Center #1 at 24 hr p.i.

Bottom mouse #2 at 24 hr p.i.

Task 2 **Months 5-6** **<u>Tarvacin derivatization with ^{74}As.</u>** We anticipate that the chemistry of any arsenic radionuclide will be identical. ^{77}As will also be the isotope of choice for radioimmunotherapy in Phase 3. Imaging based on Positron Emission Tomography (PET) will require ^{72}As or ^{74}As. Ultimately, ^{72}As would be the isotope of choice for clinical applications, but ^{74}As has a much longer half-life making it ideal for investigations in animals. Since the only source of ^{72}As or ^{74}As is currently in Europe, the long half-life of ^{74}As ($t_{1/2}$ ~ 18 days) is particularly valuable to ensure efficient use following transportation. We will verify that methods developed with ^{77}As apply to ^{74}As. Biodistribution will be assessed in each of six tumor bearing mice at 2 different times to confirm consistent behavior with ^{77}As labeled Tarvacin. (12 mice).

We had postponed ^{74}As studies since we were promised supplies from the DOE reactor at Los Alamos National Laboratory: see appended letter form Dr. Wolfgang Runde, Isotope Program, Manager, U.S.

Department of Energy. Unfortunately, the laboratory finally informed us in December '08 that they would not after all be able to ship us any 74-As. We will therefore return to the original plan of obtaining shipments from Europe. Dr. Hermanne from Brussels has confirmed his willingness to provide 74-As, as per attached letter. While frustrating, the delay is not entirely disadvantageous, since UT Southwestern has made major advances in radiochemistry facilities and PET/CT during 2008. We are now far better equipped to undertake the radiolabeling and tomographic imaging. In fall 2008 a new Siemens Inveon PET/CT instrument arrived and was commissioned. Dr Sun also occupied new state of the art radiochemistry labs, which we believe we will greatly accelerate our research and achieve the original goals during the requested 1 year no-additional cost extension.

Phase 2 Detection of diverse primary breast tumors and evaluation of metastatic spread using radio arsenic labeled Tarvacin.

Task 3 Months 7-12 <u>Determine ability to detect human breast tumor xenografts in nude mice.</u> MDA-MB-231-Luc (luciferase expressing) and MDA-MB-435-Luc cells will be implanted in mammary fat pad (MFP) of mice and allowed to grow. Growth will be monitored using calipers and bioluminescence imaging (BLI). When tumors reach 0.5 cm diameter ^{74}As-SATA-Tarvacin (100 μCi) will be administered IV to half the mice and distribution of agent will be assessed every 12 h using planar γ-scintigraphy. After 60 h small animal PET will be used to quantify biodistribution *in vivo*. At this stage, the dosed mice will be sacrificed to assess biodistribution *ex vivo*. The remaining animals will be further monitored by BLI and are expected to develop lung metastases, which will be visualized by BLI. Once metastatic spread is apparent (typically, when primary tumors reach ~ 1 cc) ^{74}As-SATA-Tarvacin will again be administered to assess the ability to detect metastases using PET. Following PET, mice will be sacrificed to determine biodistribution *ex vivo* and examine extent of metastases histologically. We will also label Rituximab, a commercial non-vascular targeting chimeric antibody, as a control (12 tumors x 2 tumor types x 2 different antibodies = 48 nude mice)

We have generated and tested highly expressing MDA-MB-231-Luc tumor cells, which will be required for the radio tracing studies and PET.

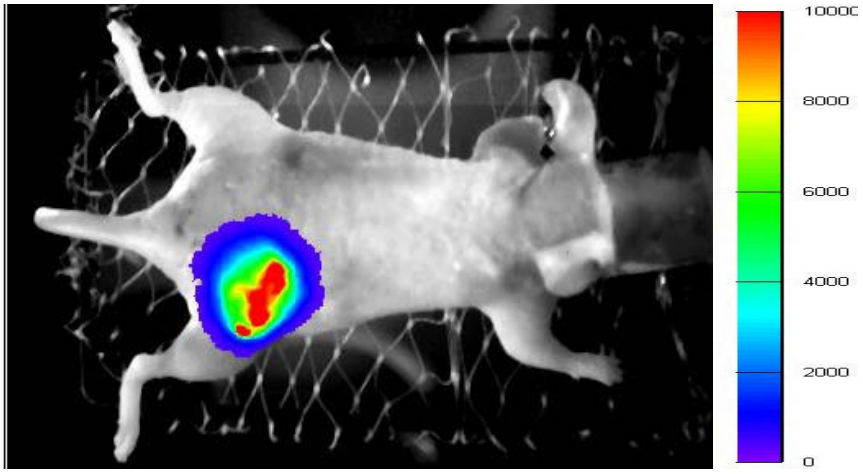


Figure 11 Bioluminescent image of MDA-MB 231 tumor growing in nude mouse

Task 4 Month 12 Prepare annual report and manuscript.

Report approved previously.

Task 5 Months 13-15 Determine ability to detect breast cancer in a bone metastasis model.

Bone metastases will be induced by intra cardiac injection of MDA-MB-231 and -435 luciferase expressing cells. Should this fail, we will implant cells directly in the femur of nude mice. Tumors will be allowed to grow and BLI will be performed twice weekly. Once BLI shows evidence for tumor growth as models of metastasis, ^{74}As-SATA-Tarvacin will be administered IV, as for Task 3 and the ability to detect the tumors will be assessed using γ-scintigraphy and PET. (6 tumors x 2 types = 12 nude mice)

Task 6 Months 16-24 Assess ability to detect syngeneic breast tumors in immuno competent rats.

13762NF and MTLn2 rat tumors will be implanted in mammary fat pad of Fisher rats. When primary tumors reach 1 cm diameter ^{74}As-SATA-Tarvacin will be administered IV and biodistribution assessed *in vivo* using PET after 24 and 60 h. Rats will then be sacrificed for *ex vivo* biodistribution and pathological examination of metastases expected in the lungs and lymph nodes, respectively. Separate cohorts of rats will be examined when primary tumors reach 1.5 and 2 cm diameter at which time metastases are expected. (6 tumors x 2 tumor types x 3 sizes = 36 rats).

Prior to extensive radionuclide imaging of rats we have conducted initial tests for proof of principle. We have examined biodistribution of ^{77}As –bavituximab in control non-tumor bearing rats. Rats survived administration and biodistribution of antibody was feasible.

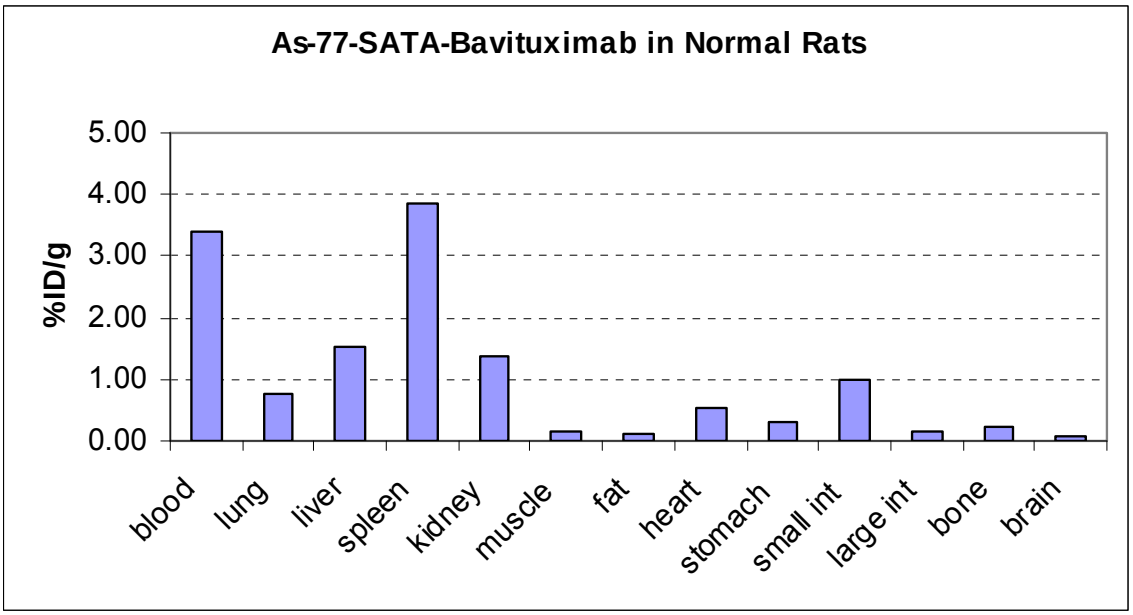


Figure 12 Biodistribution of [^{77}As]SATA-bavituximab in rat

As proof of principle [^{77}As]SATA-bavituximab was injected IV in non-tumor bearing rat with sacrifice at 4 hr. Thorpe has previously shown that PS is exposed in essentially every tumor type investigated (2). Prior to nuclear imaging studies we tested for exposure of PF in rat breast tumor (Figure 13). The panels show section of 13762NF syngeneic rat breast tumor form a Fisher rat. Bavituximab or C44 were infused into tumor bearing rats and tumors excised 24 h later. Prior to excision anesthetized rats were perfused with saline to remove unbound antibody. Panels a and d show tumor vasculature based on CD31- staining. Panels b and e show retention of antibody. B shows extensive bavituximab uptake, which is seen to be associated with blood vessel in the overlay image c. C44 was not detect. C and f show overlays and inclusion of DAPI staining for nuclei. Frozen sections were prepared and fixed with acetone. Mouse anti-rat CD31 and biotin labeled goat anti-human antibodies were used and followed by cy2 labeled streptavidin and cy3 labeled goat anti-mouse

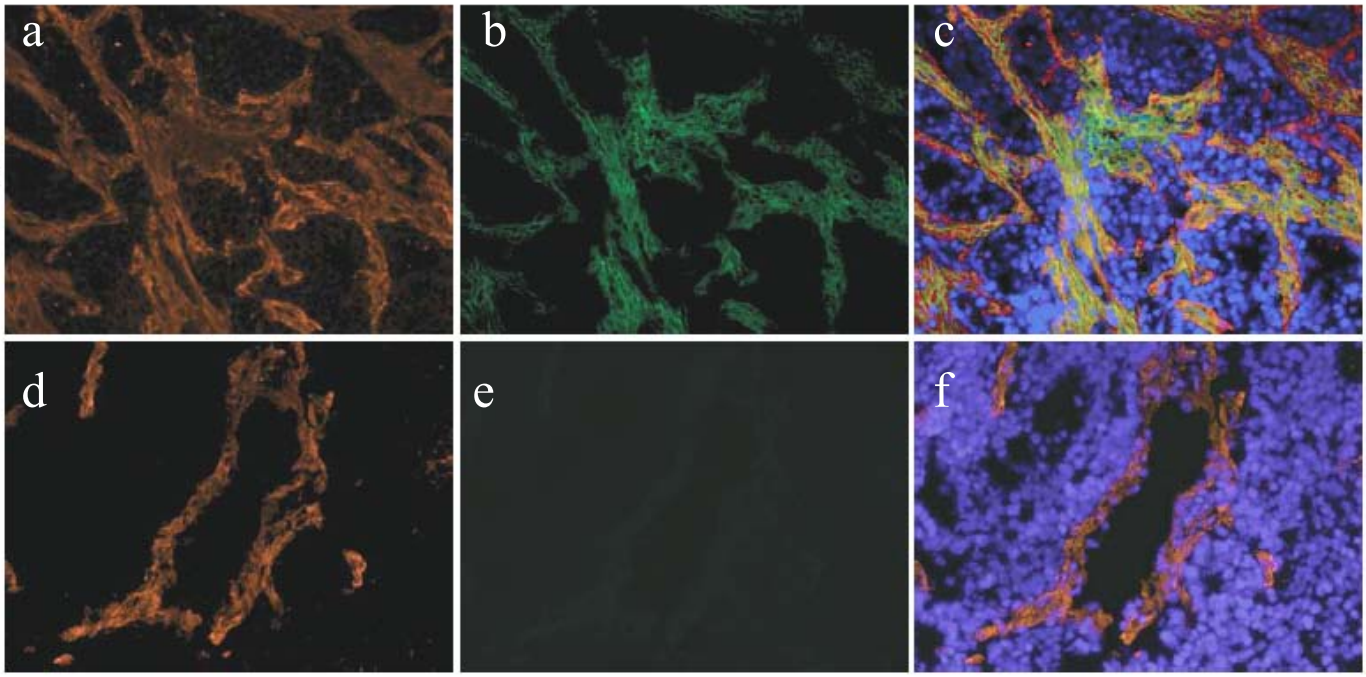


Figure 13 Expression of PS in 13762NF rat tumor 13762NF

Task 7 **Month 24** **Prepare annual report and manuscript.**

Report provided last year.

Phase 3 **Evaluate Tarvacin based radio immunotherapy of breast tumors.**

Task 8 **Months 25-30**

Assess response to single dose RIT using ^{77}As-SATA-Tarvacin. Implant 231-Luc cells in m ammary fat pad of mice. When tumors reach 0.5 cm diameter infuse single do se of either 200 or 400 μCi ^{77}As-SATA-Tarvacin. Assess growth based on calipers (prim ary tum or) and bioluminescence im aging (prim ary + m etastases). Sacrifice mice when tumors reach 1.5 cm diameter. Compare growth rates with control untreated tumor bearing mice. Com pare prevalence of m etastases at autopsy. Investigation will be repeated with MD A-MB-435-Luc tumors. (2 tumor types x 6 tumors x 3 doses (control and two doses) = 36 mice)

Investigations to be undertaken duding proposed no-cost extension.

Task 9 Months 24-34 <u>Compare response to repeat dose RIT using ^{77}As-SATA-Tarvacin.</u>

Implant luciferase transfected 231-Luc cells in mammary fat pad of mice. When tumors reach 0.5 cm diameter divide into separate groups.

i) infuse ^{77}As-Tarvacin twice weekly for 3 weeks and assess growth, as for Task 8.

ii) infuse single dose ^{77}As-SATA-Tarvacin as for Task 8, followed by non radioactive Tarvacin at therapeutic dose (100 μg/kg) twice weekly for three weeks.

iii) infuse Tarvacin at therapeutic dose (100 μg/kg) twice weekly for three weeks. Assess growth based on calipers (primary tumor) and bioluminescence imaging (primary + metastases). Sacrifice mice when tumors reach 1.5 cm diameter. Compare growth curves with control untreated tumor bearing mice. Compare prevalence of metastases at autopsy. Investigation will be repeated with MDA-MB-435-Luc tumors. (2 tumor types x 6 tumors x 3 treatments mice = 36 mice)

Investigations to be undertaken duding proposed no-cost extension.

Task 10 Months 34-36 Prepare manuscripts and final report.

3rd year report provided here in place of final report, since request has been submitted for no- coat extension.

KEY RESEARCH ACCOMPLISHMENTS:

- Improved yield of isolated ^{77}AS
- Opened new state of the art radiochemistry laboratories with additional fume hoods, lead shielding and adjacent animals housing.
- Commissioned new commercial PET/CT: Siemens Inveon PET/CT Multimodality System
- Purchased and installed new optical imaging instruments with vastly superior sensitivity: Caliper Xenogen Spectrum and Lumina which will allow detection of smaller tumor burdens for comparison with PET and assessment of metastatic tumor spread.

Figure 14: New radiochemistry facility at UT Southwestern: top left shielded hoods; top right hot cell; lower left PET/CT.

REPORTABLE OUTCOMES during year 3: Manus cript subm itted reg arding enhanced procedur e f or isolating 77As (appended):

"Separation and purification of no carrier added arsenic from bulk amounts of germanium being adequate to radiopharmaceutical labeling chemistry", M. Jahn, V. Radchenko, D. Filosofov, H. Hauser, M. Eisenhut, F. Rösch, M. Jennewein, Applied Radiation Isotopes, submitted 2009

CONCLUSION: We have verified the ability to generate and isolate ^{77}As based on activation of germanium oxide in a nuclear reactor. The current yield is lower than anticipated and we are examining methods of increasing the yield. We secured a new supply from MURR, which is able to generate much higher doses of ^{77}AS. We have demonstrated the ability of bavituximab (Tarvacin) to localize in rat breast tumor, as detected by immuno histochemistry, which is a crucial foundation for *in vivo* studies in rats. These new results provide a foundation indicating that the project will be successful.

We have requested a 1 –year no- additional cost extension. There were several reasons for delays in the investigations: i) we had hoped to obtained supplies of the arsenic-74 from the Los Alamos National Labs. This would potentially have saved funds (the US dollar had slipped severely in value), kept funds in America, eased logistic issues with Homeland Security re importation of isotopes. We were surprisingly informed in December 2008 by Donna Smith, PhD Isotope Program, LANL Program Manager that this would not after all be possible. We have again contacted Dr. Hermanne, Director of the Cyclotron in Brussels, who is willing and eager to supply us with ^{74}As. Our Office of Environmental Health and Safety has verified with Federal Express that transportation and importation will be feasible. A new letter is appended with overall cost quite similar to that quoted in the original grant application in 2005; ii) a new radiochemistry laboratory has been built, equipped and furnished to enhance investigations; iii) a new Siemens Inveon PE/CT was purchased, installed and commissioned during winter 2008/9, which will enhance the sensitivity and resolution of PET and allow correlation with anatomical CT.

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APPENDICES:

1 Letters explaining delay in undertaking ^{74}As PET investigations.

2 "Separation and purification of no carrier added arsenic from bulk amounts of germanium being adequate to radiopharmaceutical labeling chemistry", M. Jahn, V. Radchenko, D. Filosofov, H. Hauser, M. Eisenhut, F. Rösch, M. Jennewein, Applied Radiation Isotopes, submitted 2009

From: Xiankai Sun
To: Ralph Mason
Date: Fri, May 9, 2008 5:02 PM
Subject: As-74 production at LANL

Dr. Mason,

I guess this is the best that we could get from LANL with Mike Welch's help. If you think the deal is acceptable, please advise how many As-74 productions are needed for your DOD BR project. I will be out of town in Knoxville TN (Siemens facility) from 05/11 to 05/15, but plan to send Wolfgang a proposal early next week.

Best,
Xiankai

>>> Wolfgang Runde <runde@lanl.gov> 5/9/2008 3:35 PM >>>
Xiankai,
I apologize for the delay in getting back to you. I am debating with LANL when we can start producing As-74. I propose to do a test irradiation in July, send you the target to confirm that the quality of the product is acceptable. The cost estimate is about per target.

I foresee you submitting a proposal that outlines the demand for As-74 for your research in response to future solicitations.

I will be in South Korea next week. I will get back to you on this topic the following week to hopefully come to an agreement.

Please could you send an e-mail to the Isotope Business Office (clinerl@ornl.gov) requesting As-74. That way your request becomes an official track number.

Regards,
Wolfgang

--

Wolfgang Runde, Ph.D.
Isotope Program, Manager
U.S. Department of Energy
Office of Nuclear Energy

Los Alamos National Laboratory
Civilian Nuclear Program Office
SPO-CNP, Mail Stop J514
Los Alamos, NM 87545
Phone: (505) 667-3350
Fax: (505) 667-9905

A Schedule

Fed. Ex./Shipping Address:

SM-30, Bikini Atoll Rd.
Drop Pt. 48 RC1 01S
Los Alamos, NM 87545

CC: Jocelyn Chafouleas

From: Xiankai Sun
To: Ralph Mason
Date: Fri, Dec 19, 2008 12:16 PM
Subject: Fwd: Re: LANL As-74

Very disappointed after such a long waiting....

>>> "Donna M. Smith" <dms@lanl.gov> 12/19/2008 12:07 PM >>>
Xiankai,

I regret to inform you that we will not be able to irradiate and ship a target to you from LANL in 2008. We were unable to find a suitable shipping container for the target that met both of our needs before the beam to the accelerator (and our irradiation capability) goes down this weekend. We will not have irradiation capability again at LANL until ~June of 2009.

I apologize that we were unable to find a suitable container and that I was not able to give you a firm answer back in June when we first discussed this. When it was determined that your facilities did not have the necessary infrastructure to use our approved shipping container, I had hopes that we would be able to identify an alternative container, but after evaluating several containers from June through December, we still could not find a container that would work for both of us. When I learned in December that our shippers were hesitant to use the latest container we thought might work, I knew there would not be time to finalize the container before we lost our opportunity to irradiate. If something changes between now and June of 2009, I will let you know.

Sincerely,

Donna Smith
--
Donna Smith, PhD
Isotope Program
LANL Program Manager
J519 Los Alamos National Laboratory
Los Alamos, NM 87544

phone: 505-667-0160
pager: 505-949-0048
email: dms@lanl.gov
cell phone: 505-629-6735

Prof. R. Mason
Professor of Radiology
University Texas Southwestern Medical Center
E6.238E
5323 Harry Hines Boulevard
Dallas, Texas 75390-

VUCY 14/09
AH/ah

29 April 2009

Dear Sir,

We are honoured to receive your request for a quotation for the production of ^{74}As.
The changes for the irradiation will be as follows.

Production of ± 37 GBq of ^{74}As by ^{nat}Ge(α,x)^{74}As reaction.
Estimated beam time 1 hour at 15μA on target, Ep= 20 MeV; cost : 900Eur

A handling and measuring fee of 120Eur will be added.
These costs are 12% overhead and 21% VAT not included.
These taxes will have to be added if an official invoice form the Vrije Universiteit Brussels is needed, but can be avoided if a simple bill for reimbursement issued by our lab is sufficient.
Payment should then be be done by transfer to a bank account of the VUB Cyclotron c/o A. HERMANNE.

The price does not include transport or shipment, which should be organised on mutual agreement and paid separately. The price for one package , a type A UN2915 cardboard box containing a Pb shielding container, would be about 600 Eur, taxes and fees not included. The colli, not to be returned, will be provided by us and are estimated at 100-120Eur a piece.
In the hope that this quotation can meet your approval and awaiting an official order stating number of irradiations wanted and optimal date of delivery.

Best regards

Prof. A. Hermanne
Head of Cyclotron Department.

Submitted Applied Radiation Isotopes

Separation and purification of no carrier added arsenic from bulk amounts of germanium being adequate to radiopharmaceutical labeling chemistry

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Keywords:

As-72, As-74, As-77, radioactive arsenic, germanium metal, radiochemical separation, labeled antibodies

Abstract
Radioarsenic labeled radiopharmaceuticals could add special features to molecular imaging with positron emission tomography (PET). For example the long physical half-lives of ^{72}As ($T_{1/2}$ = 26 h) and ^{74}As ($T_{1/2}$ = 17.8 d) in conjunction with their high positron branching rates of 88 % and 29 % respectively, allow the investigation of slow physiological or metabolical processes, like the enrichment and biodistribution of monoclonal antibodies (mab) in tumor tissue or the characterization of stem cell trafficking. A method for separation and purification of no carrier added (nca) arsenic from irradiated metallic germanium targets based on distillation and anion exchange is developed. It finally converts the arsenic into an *As(III) synthon in PBS buffer and pH 7 suitable for labeling of proteins via As-S bond formations. The method delivers radioarsenic in high purity with separation factors of 10^6 from germanium and an overall yield from target to labeling synthon of > 40%. In a proof-of-principle experiment, the monoclonal antibody Bevacizumab, directed against the human VEGF receptor was labeled with a radiochemical purity > 90% within 1 h at room temperature with nca $^{72/74/77}$As.

1. Introduction

Arsenic:
The element arsenic is well known as the favorite poison of the Savellis, the Borgias and Agatha Christie [1]. There were also speculations that Napoleon Bonaparte died because of arsenic poisoning [2]. Because arsenic is viewed as synonymous with toxicity it is still used today for homicides or suicides [3]. A worldwide problem is the local high pollution of natural water with arsenic under natural conditions or as a result of additional human pollution [4]. Therefore the removal of arsenic from drinking water is an important challenge.

Despite its toxic properties arsenic was used 2000 years ago by Greek and Chinese healers as therapeutic agent to treat everything from syphilis to cancer [1]. More recently, the use of arsenic trioxide (ATO) in European medicine increased after the invention of Fowler's solution which was used for a number of systemic illnesses from 18th to 20th century. Over the last 100 years, the concerns about toxicity and potential carcinogenicity of arsenic administration have declined its use for medical application [5]. At the end of the last century it was found that ATO has very good therapeutic characteristics for treatment of acute promyelocytic leukemia (APL) and clinical trials have been confirmed all around the world [6, 7]. Some years ago ATO was approved by the U.S. Food and Drug Administration (FDA) as Trisenox® for this indication [8].

Another application of arsenic radioisotopes might be its use as radioactive probe in sub-toxic trace amounts for biological or medical purposes. The tracer concept of radiopharmaceutical chemistry allows the application of no carrier added (nca) amounts of radioactive isotopes (e.g. of arsenic) that are used for labeling of interesting biological carriers like monoclonal antibodies (mab) and the imaging of their biological behavior *in vivo*. The element arsenic provides a range of isotopes for non-invasive PET imaging like ^{72}As ($T_{1/2}$=26 h; 88% β^+), and ^{74}As ($T_{1/2}$=17.8 d; 29% β^+). These isotopes are produced by (p,n)-reactions on natural or isotopically enriched targets of metallic germanium or germanium(IV)oxide and are therefore available in nca state. Additionally, the β^- emitter ^{77}As ($T_{1/2}$=38.8 h; 100 % β^-) is produced by bombardment of the same target materials with neutrons, followed by β^- decay of ^{77}Ge ($T_{1/2}$=11.3 h; 100% β^-).

Separations

Various methods for the separation of germanium and arsenic have been reported so far. A collection of separating procedures for germanium and arsenic from fission products is given elsewhere [9-11]. The majority of these methods is based on distillation of germanium as $GeCl_4$ followed by distillation of arsenic as $AsCl_3$. Other methods were based on solvent extraction or precipitation [12] of one of the two elements. Most of these methods fail for radiopharmaceutical application because arsenic carrier was used, which is not in common with the tracer concept. A review on the chemical behavior of radio germanium is given by Mirzadeh et al. [13], involving separation techniques for radioarsenic. Separation of As(III) from antimony and bismuth was achieved in 8 M HCl and extraction into benzene [14]. A systematic study on separation of As(III) and Ge(IV) in HCl and HI systems by liquid liquid extraction into several solvents was carried out by Brink et al. [15]. An easy separation of arsenic from germanium was achieved by solvent extraction of arsenic from sulfuric acid solutions after addition of KI into toluene [16, 17]. Another strategy for the separation of As from GeO_2 in HCl is the oxidation of As to As(V) and extraction of the Ge into organic solvents [18-20]. After the separation the As(V) is reduced again to As(III) and extracted into organic solvents. Tolmachev et al. [21] processed a germanium(IV)dioxide target after proton bombardment by dry distillation followed by wet chemistry workup for additional purification. The low target loss of less than 1 % was mentioned as far as a further target recovery is not needed. Some methods based on distillation of $GeCl_4$ are reported whereas arsenic was kept in the nonvolatile form of As(V) [22] followed by solvent extraction [23, 24], cation exchange [25] or anion exchange [26]. Schindewolf et al. [27] absorbed Ge and Ga from diluted HF solutions on anion exchange resin AG 1X8 while As(III) was not absorbed. Some more data about the behavior of As(V) in HF media [28] and mixtures of HF/HNO_3 media [29] on AG1X8 is available although data for Ga differ in [27] and [28]. The behavior of Ge, As(III) and As(V) in HCl media on AG1X8 is very well known from literature [30-32] and some data about the absorption in HCl/acetone media is available [33]. Ge and As(III) are strongly absorbed at high HCl concentrations and can easily be separated from As(V) which shows only slight absorption. Korkisch et al. [34] used the same resin, but HCl/acetic acid medium for separation of Ge and As(III)/As(V). Arsenic behavior on cation exchange resin is determined by very low absorption coefficients in HCl and $HClO_4$ media [35]. A slightly higher absorption was found in HCl/acetone media [36] and in HBr media [37]. Jennewein et al. [38] developed a separation method for GeO_2 targets based on solid phase extraction after dissolving the target in HF_{conc}. The radioarsenic was fixed on a BOND ELUT ENV cartridge after treatment with NaI while the GeF_6^{2-} formed in HF passed without interaction through the column. After drying the cartridge with nitrogen gas the radioarsenic was eluted with ethanol. This method so far is the only one that lead to a radioarsenic labeled mab [39] that was used for tumor imaging with PET *in vivo* [40].

There are also some interesting approaches that are far-out the normal standard techniques. Maki et al. [41] developed a separation technique for ^{77}Ge, ^{77}As(III) and ^{77}As(V) by TLC with and without the addition of arsenic carrier. Caletka et al. [42] developed a separation technique based on the absorption of germanium in 8 M HCl or other mineral acids on silica gel columns. Under these conditions elements like gallium, arsenic and zinc are eluted from the column with 8 M HCl whereas germanium stayed on the column.

Targetry

Preliminary tests for the production of radioarsenic isotopes on GeO_2-targets at high flux reactors and cyclotrons showed for long term irradiations of 1 week (reactors) or high proton beam currents (more than 2 µA) that, following dissolution in HF_{conc}, appreciable amounts of the target material formed insoluble compounds as a consequence of thermal stress and radiolysis. This insoluble compound was identified as elemental germanium. However, aiming for medical application of radioarsenic isotopes, high neutron fluxes or high proton

beam currents need to be applied on the targets for high production yields. Therefore germanium metal of natural composition was chosen as target material.

Approach

The aim of this work thus was to set up a procedure for separation of nca amounts of radioactive arsenic isotopes from macroscopic amounts (100 - 200 mg) of irradiated metallic germanium. The desired high purity of the final radioarsenic fraction was intended by crude separation of germanium by distillation of $GeCl_4$ followed by anion exchange chromatography to remove the remaining trace amounts of germanium. Additionally, a separation of radioactive contaminations (gallium and zinc isotopes formed during neutron bombardment [43], namely $^{72/73}$Ga and ^{69m}Zn) was considered. In cyclotron irradiations, the major contamination found was ^{67}Ga. The separation of those contaminations is very important as far as a radioarsenic based tracer for possible human use must be available in highest radiochemical purity. The combination of a distillation technique with an anion exchange column has been used in a similar setup before [26]. This method lead to a high purity *As(V) fraction in 10 M HCl not feasible for labeling of biomolecules. The challenge for the labeling of mab is first to reduce the *As(V) to *As(III) and second to remove the 10 M HCl as far as mab labeling requires a pH of around 7. For aspired in vivo experiments the final product needs to be concentrated to low volumes of about 500 µl or less.

3. Materials / Methods

Materials

If the radioarsenic is used for labeling chemistry of proteins in nanomolar concentrations care must be taken that the addition of cold arsenic carrier by used chemicals is minimized. This is also the case as long as the radiolabeled product shall be used in a clinical environment. Therefore all chemicals were purchased in the highest purity available, e.g. suprapure acids. Germanium metal (99.999%) was purchased from Goodfellow as a plate of 50x50x0.5 mm and laser-cut into discs of 9.9 mm diameter (204 mg each) and used for cyclotron irradiations. For reactor irradiations, germanium metal (99.9999%) was purchased from Chempur in small pieces ranging from 100 - 200 mg. Concentrated nitric acid (65 %, Suprapur) and hydrochloric acid (30 %, Suprapur) were purchased from VWR. CuCl (99.995+%) and chloroform (analytical grade) were purchased from Sigma Aldrich. Anion exchange resin AG1X8 (200 - 400 mesh) was purchased from BioRad. PBS buffer and TCEP (tris(2-carboxyethyl)phosphine) were purchased from Pierce.

Proteins

The monoclonal antibody Bevacizumab was chosen as a model protein for the demonstration of the ability of this method to provide radioarsenic in a form suitable for labeling of SH group bearing molecules. The Bevacizumab was purchased from LaRoche at a concentration of 25 mg/ml. Bevacizumab is directed against the VEGF receptor and used as anti cancer agent in human therapy [44, 45]. Each labeling experiment was performed by using 50 µl (corresponding to 1.25 mg) of the original antibody solution. The solution was filled up with 450 µl of PBS buffer and added to the radioarsenic solution.

Irradiations

The positron emitters ^{72}As ($T_{1/2}$=26 h; 88% β^+) and ^{74}As ($T_{1/2}$=17.8 d; 29% β^+) were produced simultaneously via (p,n)-reactions on ^{nat}Ge targets at the German Cancer Research Center (DKFZ) cyclotron MC32NI in Heidelberg. Cross sections for the production of positron emitting arsenic isotopes were measured by Spahn et al. [46]. Detailed decay properties of

arsenic isotopes can be found elsewhere [38, 47, 48]. For the production of positron emitting arsenic isotopes (^{72}As and ^{74}As) germanium discs of 9.9 mm diameter and 0.5 mm thickness (204 mg) were placed into an aluminum container and covered with a 50 µm Harvard foil. The beam current was up to 30 µA with 15 MeV protons and irradiation time was diversified from 0.5 to 5 h. Integrated loading was up to 100 µAh. This setup showed no destruction of target material. As far as Ge targets of natural composition were used ^{70,71,72,73,74,76}As are produced via (p,n) reactions. Major isotope after 1d cooling time is ^{72}As. If the target was allowed to cool for 1 week the major isotope is ^{74}As with small contaminations of $^{71/73}$As. In a cyclotron irradiated target any Ge isotopes formed are not useful for γ-ray spectroscopy due to undesirable low cross sections. The system ^{77}Ge/^{77}As provides a unique option for detection of both elements simultaneously. The nca isotope ^{77}As ($T_{1/2}$ = 38.8 h; 100% β^-) was produced by (n,γ)-reaction on ^{nat}Ge target in a nuclear reactor. It was used for optimization of the chemical separation procedure, determination of yields and for the optimization of labeling chemistry. Detailed cross sections for nuclear reactions of neutrons on germanium can be found elsewhere [43]. The production of ^{77}As was carried out by irradiation of 100 - 200 mg germanium metal pieces for 6 h at a neutron flux of $4.2 \cdot 10^{12}$ n cm^{-2} s^{-1} at the TRIGA Mark II reactor of the Institute of Nuclear Chemistry, Mainz. The target was allowed to cool for 18 h to form ^{77}As from decay of ^{77}Ge and used directly for separation. Therefore the separation factors have been measured in this system and the same procedure was then applied to the positron emitting, cyclotron produced arsenic isotopes.

Radiochemical separation

Dissolving the target: The germanium target (100-200 mg) was placed in a quartz distillation apparatus (Fig. 1) and 4 ml *aqua regia* was added. The apparatus was heated to 120°C and during this time the irradiated germanium metal dissolved.

Distillation of $GeCl_4$: After complete dissolution of the target the temperature was maintained at 120°C for distillation of $GeCl_4$. For acceleration of this process a stream of Argon was bubbled through the solution. Over a period of 1.5 h additional 6 ml of HCl (10 M) were added. The $GeCl_4$ was trapped in an ice cooled vessel containing 20 % H_2SO_4. After completed clearance the solution was condensed to less than 500 µl.

Anion exchange: The distillation solution was filled to 500 µl with 10 M HCl. The solution was transferred onto an anion exchange column (3*100 mm, AG1X8) in the chloride form and eluted with 500 µl fractions of 10 M HCl. Arsenic *As(V) was eluted in the fractions 2 and 3. After 10 fractions the eluent was switched to 0.1 M HCl for removal of gallium, germanium and zinc isotopes.

Reduction of As(V) to As(III), extraction into CCl_4 and back extraction into PBS-buffer: Fractions 2 and 3 were combined (1 ml solution) and mixed with 50 mg CuCl. The mixture was heated at 60°C for different periods ranging from 5 to 120 minutes, with 60 minutes finally applied for the batch experiments. The As(III) was extracted twice with 500 µl CCl_4. Combined CCl_4 fractions were extracted with 500 µl PBS-buffer containing 25 mM EDTA and 0.5 M hydroxylamine.

Speciation of As(III) and As(V): The oxidation state of the radioarsenic in the CCl_4 and the PBS fraction was determined by radio TLC. 0.5 µl of each fraction was spotted on a Si-60 silca plate and developed with 0.01 M sodium tartrate / methanol 3 : 1. TLC plates were analyzed using an Instant Imager from Packard.

Determination of radiochemical purity and radiochemical separation yields: The radiochemical purity and separation yield were determined by γ-ray spectroscopy. The activity of the undissolved Ge target and of 500 µl aliquots of the solution after each step were measured in the same geometry and compared quantitatively. The γ-ray spectroscopy was performed using an Ortec HPGe detector system and Canberra Genie 2000 software. For the ^{77}Ge/^{77}As-system, the two nuclides were determined by their most abundant γ-lines of 239 keV (1.6 %) for ^{77}As and 264 keV (53.9 %) for ^{77}Ge. As long as ^{77}Ge has a much higher gamma emission rate compared to ^{77}As it is clearly visible in the target spectrum but could not be detected in the purified fraction. Care was taken that the dead time of the detector remained always below 10 %. The irradiated target was measured in 1 m distance to the detector for 15 min, whereas the purified fraction was measured for 12 h directly on the surface of the detector. All data were normalized to the time point of the first acquisition by the acquisition software.

Labeling of Bevacizumab: The 500 µl of the purified radioarsenic solution in the PBS fraction was combined with 500 µl of Bevacizumab solution (1.25 mg, 8 nmol). 10 µl of TCEP (10 mg/ml, 420 nmol) were added and the mixture was allowed to stand at room temperature for 1 h. At various time points aliquots were taken for analysis and detection of labeling yields via gel filtration HPLC (HiTrap desalting column) or radio TLC.

4. Results/Discussion

Irradiations

Under the chosen conditions, the neutron irradiations at the TRIGA Mark II reactor Mainz resulted in about 4 MBq ^{77}As and about 2 MBq ^{77}Ge at beginning of target workup (18 h EOB) for a 150 mg Ge target. The ^{72}Ga ($T_{1/2}$=14.1 h) and ^{69m}Zn ($T_{1/2}$=13.9 h) were produced in low yields due to low cross sections for fast neutron reactions of about 4 kBq (^{72}Ge(n,p)^{72}Ga) and about 1 kBq (^{72}Ge(n,α)^{69m}Zn). The production yield for the positron emitting isotopes of arsenic under the chosen conditions (15 MeV protons) was about 1 MBq/µAh for ^{74}As and 6 MBq/µAh for ^{72}As. The ^{67}Ga ($T_{1/2}$ = 78.3 h) was produced by ^{70}Ge(p,α)^{67}Ga reaction (about 35 kBq/µAh).

Dissolving the target

Although germanium metal is slowly attacked by aqua regia [13] it was chosen as solvent. The nitric acid oxidizes the Ge(0) to Ge(IV) which is precipitating as GeO_2 in the HCl. The dilution of the target accelerates with increasing temperature. Under these oxidative conditions the n.c.a. radioarsenic is also oxidized to * As(V). As far as the subsequent distillation requires about 120 °C, the germanium is dissolved within 30 min which are required for heating the solution. Dissolving of metallic germanium target results in a milky solution of germanium(IV)oxide.

Distillation of $GeCl_4$

After dilution of the target the temperature is maintained at 120 ° C to distill the $GeCl_4$ (b.p.=84 °C). Additionally a slight stream of Argon was bubbled through the solution to accelerate the distillation. Under these conditions the radioarsenic stays in oxidation state

*As(V) which is not volatile. In contrast to other publications [9], no other reagents were added to keep the arsenic in the pentavalent oxidation state. During 1.5 h distillation period a total volume of 6 ml 10 M HCl was added periodically for distillation of $GeCl_4$ and destruction of the remaining nitric acid. Traces of nitric acid will interfere in later reduction of *As(V) to *As(III) and lead to low extraction yields. Finally the solution is condensed to less than 500 µl and taken out of the distillation apparatus. An average loss of arsenic of 10 % was observed in this step. It is basically dedicated to absorption on the walls of the glassware. In this step an average separation factor of germanium of $2*10^4$ was achieved. Care should be taken to avoid condensation of $GeCl_4$ on the surface of the vessel which might give some additional contamination of the solution with Germanium. The non-arsenic radionuclides produced during irradiation at cyclotron and reactor (namely $^{67/72}$Ga, ^{69m}Zn) cannot be removed by distillation and remain inside the arsenic solution.

Anion exchange purification

According to the behavior of arsenic, germanium, zinc and gallium on anion exchange resin AG1X8 in HCl [30-32], *As(V) is only slightly retained compared to Ge, Zn and Ga at high HCl concentrations. Consequently, 10 M HCl was chosen to elute *As(V) from the column. The *As(V) is eluted in fraction two and three, containing > 90 % of arsenic activity in 1 ml of 10 M HCl. Ge, Zn and Ga are only eluted after changing the eluent to 0.1 M HCl. A typical elution profile for *As(V), *Zn(II) and *Ga(III) is given in Fig. 2. However, according to [30] Ge is eluted at HCl concentrations < 5 M and is strong retained at 10 M HCl with a distribution coefficient of about 200 compared to 4 of As(V). For determination of the final radionuclidic purity an aliquot of the combined fractions two and three was measured for 12 h at the closest position of the detector. Any ^{77}Ge activity could be observed. As the detection limit for this setup was determined to be 0.25 Bq ^{77}Ge and the experiment started from 2 MBq of ^{77}Ge, an overall separation factor for the anion exchange column of ^{77}Ge/^{77}As of > 10^6 is considered.

Reduction of As(V) to As(III), extraction into CCl_4 and back extraction into PBS-buffer

Batch experiments showed that both NaI and CuCl are suitable reducing agents for *As(V) to *As(III). In the case of of NaI with nca activities of *As always re-oxidation to *As(V) was observed in CCl_4 by co-extraction of I_2 formed. Therefore CuCl was used which did not show comparable effects and delivered *As(III) in CCl_4. Reduction of *As(V) was achieved in high yields by heating the solution for 1 h at 60°C. Twice extraction of As(III) in 500 µl CCl_4 gave an average yield of > 70%. This offers the ability to label molecules in non aqueous medium. Back extraction was performed by extraction the combined organic phases with 500 µl PBS buffer in average yield of > 60%. The overall yield was 40 %. Extraction yields might be upgraded by repeated extraction. However, the aim was also to minimize the final volume to about 500 µl PBS buffer to achieve high concentrated solutions of arsenic activity.

Speciation of As(III) and As(V)

One of the key steps for labeling of mab with arsenic isotopes is the availability of *As(III) in the labeling solution. This was monitored by a radio TLC method. While mixtures of HCl/acetone [13, 41] lead to destruction of the TLC plate, a mixture of sodium tartrate and methanol was chosen. The R_f values of 0.9 for *As(V), 0.6 for *As(III) and 0 for *As-labeled mab (see Fig.3) are comparable to those found in HCl/acetone mixtures [41]. Care should be taken that the separated samples are used for labeling immediately or stored frozen in an

atmosphere of argon to prevent the *As(III) from re-oxidation. The final fraction in 500 µl PBS contained > 95 % *As(III).

Labeling of Bevacizumab

The principle labeling strategy for radioarsenic is based on its high affinity to free SH-groups. The thiol-free reducing agent TCEP reduces some disulfide bonds inside the mab that will subsequently react with the *As(III), obtained from the separation method. To prevent a loss of immunoreactivity of the mab, a low concentration of TCEP (420 nmol) was used as mentioned in the instruction manual [49]. TCEP alone shows no interaction with arsenic and thus can stay inside the solution during the reaction. After 1 h at room temperature the labeling yield was > 90 % determined by TLC and > 99 % by gel filtration HPLC (Fig.4). The antibody can be purified by gel filtration from the excess of free TCEP.

5. Conclusion

A highly selective method for the separation of nca radioarsenic isotopes from bulk amounts of germanium targets and trace contaminations of Zn and Ga radioisotopes was developed. The method can be applied to natural germanium targets or isotopically enriched target material irradiated at nuclear reactors or cyclotrons. It is also possible to use GeO_2 as target material. Nca radioarsenic and macroscopic germanium are separated in a two step procedure. Metallic germanium and As(V) are first separated by distillation with an average separation factor of $2* 10^4$. This is followed by purification with anion exchange chromatography for separation of the remaining germanium and radioactive trace amounts of Zn and Ga formed during irradiation. The overall separation for germanium/arsenic was > $1•10^6$. In order to apply the separated radioarsenic for labeling of proteins, the *As(V) was reduced to *As(III) with CuCl at elevated temperature within 1 h. As far as the labeling of mab requires neutral pH conditions, the *As(III) was first extracted into CCl_4 and then back extracted into PBS buffer. The overall yield of *As(III) from the target to the final 500 µl PBS fraction is > 40%.

Labeling of antibodies was successfully exemplified with the monoclonal antibody Bevacizumab providing labeling yields of > 99 % after 1 h incubation at room temperature. This demonstrates that the radiochemical separation procedure is not only effective in terms of radiochemical parameters, but also adequate to the application of radioarsenic for syntheses of relevant protein based radiopharmaceuticals.

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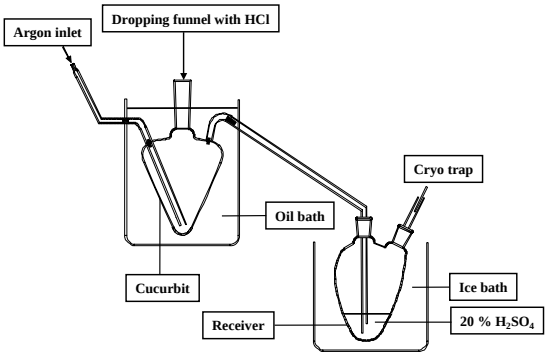

Fig. 1: Schematic drawing of distillation apparatus

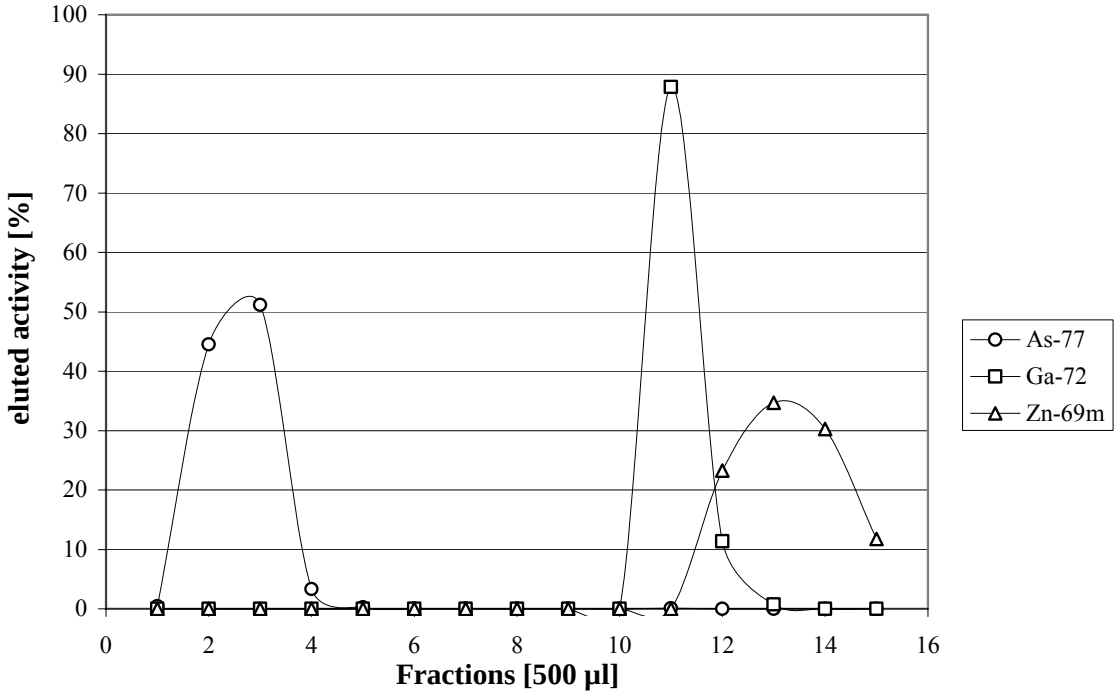

Fig. 2: Elution profile of ^{77}As(V) distillate for reactor irradiated Ge target. As(V) is eluted in fraction 2 and 3 in 10 M HCl. Eluent was changed to 0.1 M HCl in fraction 11. Under these conditions Ga and Zn isotopes are eluted from the column.

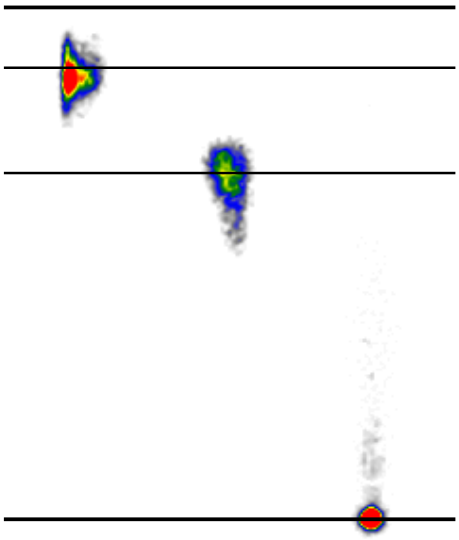

Fig. 3: Radio TLC of *As(V), *As(III) and *As labeled mab

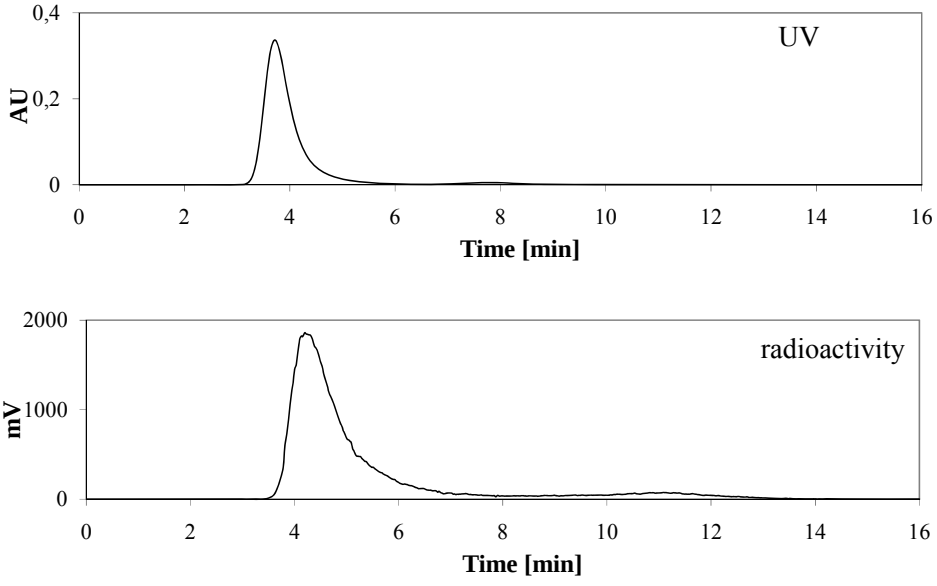

Fig. 4: Gel-filtration chromatogram of ^{77}As-Avastin after 1 h (black: UV 280 nm, blue: radiosignal)

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